The potential and pitfalls of exploiting nitrogen fixing bacteria in agricultural soils as a substitute for inorganic fertiliser*

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ABSTRACT

Nitrogen fixing bacteria have been used for centuries to improve the fertility of agricultural soils. Since the introduction of inorganic nitrogen (N) fertiliser that provides a reliable boost to crop yields whilst reducing land and labour requirements, the use of biological nitrogen fixation has been in decline. Recently, concerns have been expressed about the sustainability of inorganic N fertiliser application, however, there remain doubts about whether N_2 fixing bacteria alone can provide agriculture with sufficient fixed N to feed a burgeoning global population. In this paper we review the current state of our knowledge regarding those diazotrophic bacteria that have a role to play in agriculture. We focus on our current areas of research, particularly, the importance of understanding the classification and mechanism of action of N_2 fixing bacteria that are used in agricultural soils. We discuss the applications of N_2 fixing bacteria that illustrate their potential to provide sustainable N, particularly focussing on Australian and South American agricultural systems where these bacteria are widely exploited to maintain soil fertility. We also identify problems with the use of bacteria as inoculants, including ineffective inoculation due to poor quality preparation, the use of appropriate isolates and issues with sustainability. We review the outlook for biological N fixation highlighting how molecular biology may enable the expression of N fixation in non-leguminous crops.

INTRODUCTION

Nitrogen is, apart from water and organic carbon, the most important nutrient that can limit crop yield in agricultural soils. As a result many soils are supplemented with nitrogen (N) to increase productivity. Historically, the addition of N relied upon the use of legume rotations, during whichthe legume would fix atmospheric N through the symbiotic association of the rhizobial bacteria that nodulate the roots of these plants. While biological nitrogen fixation (BNF) using leguminous crops can fix substantial amounts of nitrogen, for example, crop legumes have been estimated to fix ~155 Kg N/ha in Australian agricultural systems (Crews and Peoples 2004), it requires a greater investment in land and labour than using inorganic fertiliser N.

As a result, there is an increasing use of inorganic fertiliser which can be rapidly applied, is cheap compared to the value of the extra crops it produces and gives more reliable boosts to crop yields. For example, in a UK experiment the application of inorganic fertiliser at 192 Kg N/ha provided an additional 5.72 tonnes of wheat compared to a corresponding plot that had no additional N added. This represented to a 485% increase in revenue from the same area of land even after additional processing costs and the price of the inorganic N were accounted for (Jenkinson 2001). For the farmer, the use of inorganic N fertiliser increases the productivity of their land as they can dispense with the necessity to grow N_2 fixing legumes and concentrate on the production of cereals or other more valuable crops (Crews and Peoples 2004).

Despite the effectiveness of inorganic N fertiliser its use does not come without a cost. In terms of the environmental impact, inorganic fertiliser is derived from fossil fuels, particularly natural gas. The increasing costs of energy has seen the price of fertiliser in the UK increase by 50% in the last 5 years (DEFRA, 2006. http://www.defra.gov.uk/). Moreover, the use of fossil fuels is not sustainable, and the production of inorganic N by the Haber-Bosch process generates huge amounts of the greenhouse gas CO_2, between 0.7-1.0 tonnes per tonne of

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ammonia. Given that 98 million tonnes of N_2 are fixed annually by this process the emission of CO_2 is significant (Jenkinson 2001). In addition, many farmers in developing countries find the costs of inorganic fertiliser prohibitive and often apply significantly less than the recommended amounts (Sanginga 2003).

Therefore, BNF remains a viable alternative to the use of inorganic N, particularly for resource poor farmers that traditionally rely on legume rotations to maintain the fertility of their soil. However, increasing populations, particularly in developing countries, are mitigating against the use of BNF as productive agricultural land comes under increasing pressure to meet the demand for food. The situation is set to deteriorate, as the major increase in population over the next 50 years will occur in the developing world, hence the food to feed these people will need to be grown in those regions. Most developing countries, have environmental constraints that will impede the development of agricultural systems able to meet this challenge. These include lack of water, desertification and insufficient cultivable land. As a result, it has been argued that current models of low input agriculture relying on BNF and requiring large areas of land will be unlikely to provide the annual requirement of an extra 15 million tonnes of protein by 2050 to stave off widespread hunger (Jenkinson 2001; Smil 2001). However, a more optimistic view has been articulated by other workers who suggest that through changes in diet, trade policies and a reduction in food wastage many countries could virtually eliminate their dependence on inorganic N fertiliser by freeing up land for legume based agriculture, increasing the overall sustainability of the global farming industry (Crews and Peoples 2004).

In any event, the future presents formidable, economic, political and scientific challenges if the population increase predicted by the middle of this century is to be provided with sustainable and secure food sources. Whether ultimately BNF will have a role in providing the additional protein required remains to be seen.

In this paper we review the current state of our knowledge regarding the bacteria that have a role to play in BNF, we focus on our current areas of research, particularly, the importance of understanding the classification and mechanism of action of bacteria that are used in agricultural soils. We discuss applications of N_2 fixing bacteria that illustrate their potential to provide sustainable N, particularly focusing on Australian and South American agricultural systems where these bacteria are widely exploited to maintain soil fertility. We also identify problems with the use of bacteria as inoculants, including ineffective inoculation due to poor quality preparation, the use of appropriate isolates and issues with sustainability. Finally we discuss the future direction and potential of bacterial mediated BNF in agricultural systems.

NITROGEN FIXING BACTERIA WITH KNOWN OR POTENTIAL APPLICATIONS IN AGRICULTURE

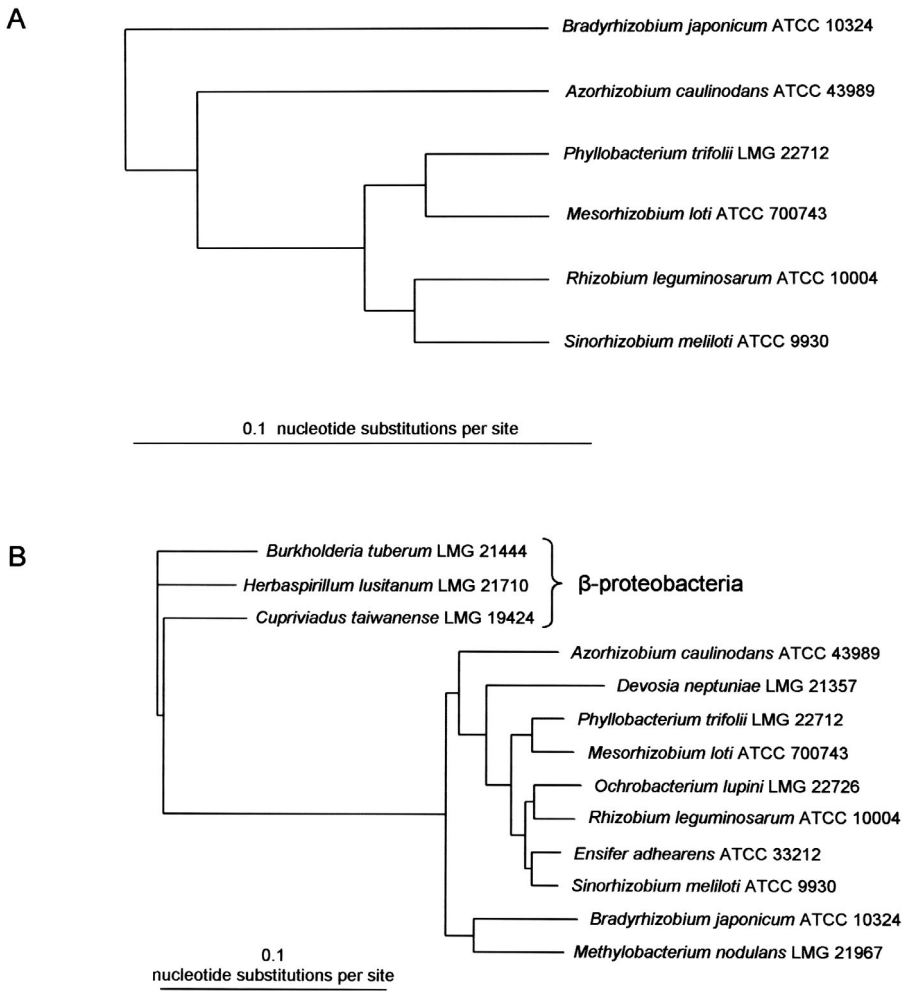
Bacteria capable of fixing N are dispersed across a wide range of taxa, however, in terms of their impact in agricultural soils they can be divided into three groups. The first are those that nodulate and form symbiotic interactions with a host plant, these are collectively referred to as the rhizobia. The second group are free-living bacteria in the soil that can potentially form associative interactions with crops. The final group are described as endosymbiotic bacteria. They are proposed to colonise plant tissue without forming specific symbiotic structures such as nodules. In the ensuing sections we shall review the current knowledge of each of these groups in terms of their classification mechanism of action and potential applications in enhancing the nitrogen status of agricultural soils.

SYMBIOTIC N_2 FIXING BACTERIA

Legumes include some of the most important commercial crops under cultivation, such as soybean (*Glycine max*), pea (*Pisum sativum*) and common bean (*Phaseolus vulgaris* L.). In Brazil, for example, soybean cultivation is expanding rapidly, particularly with the introduction of genetically modified varieties. In addition, the common bean is an important staple crop, over 5.5 million hectares are grown annually providing 30% of the populations protein requirement (Hungria et al. 2000). In Europe, there is also significant scope for increased production and utilisation of grain legumes such as lentils (*Lens culinaris*), white lupin (*Lupinus albus*) and chickpea (*Cicer arietinum)*. Clovers (*Trifolium* spp) and alfalfa (*Medicago sativa*) are also used widely as forage crops in agricultural systems which are based on livestock production (Carlsson and Huss-Danell 2003). Traditionally, legume rotations were widely used to enhance soil N but in recent years these have been replaced through the application of inorganic N fertiliser. However, an increased emphasis on sustainable agricultural production suggests that the use of symbiotic N fertilisation will continue to make a significant contribution to the N budget of agricultural soils. Therefore, the bacteria that form symbiotic relationships with leguminous plants, producing root nodules, will continue to provide the most significant contribution to BNF in the agricultural context.

These bacteria are collectively referred to as the rhizobia, however, they do not belong to a phylogenetically coherent group. The rhizobia have been the subject of much debate over their taxonomic status in recent years, six years ago the rhizobia comprised seven genera all in the family Rhizobiaceae within the a subclass of the phylum Proteobacteria. This phylogeny was based on 16S rRNA gene analyses, however, there were a number of problems, not least that the phylogeny based on this analysis meant that species of the rhizobial genus *Rhizobium* were interdigitated on the phylogenetic tree with those of a genus of plant pathogens called *Agrobacterium*. Young et al. (2001) showed that the genus *Rhizobium* could not be differentiated on the basis of 16S rRNA phylogenetic analyses from the *Agrobacterium* and proposed that they were merged. Subsenquently after this publication the

Fig. 1. Unrooted phylogenetic trees based on 16S rRNA gene sequences showing the recent changes in the taxonomy of the rhizobia, each genus is represented by a representative type strain. (A) The classification of the rhizobia after Young et al. (2001) into six genera of α-Proteobacteria. (B) The current classification of the rhizobia reflecting the identification of more species capable of nodulating legumes including representatives from three genera of β-Proteobacteria. The scale bar represents the number of nucleotide substitutions per 10 nucleotides.



rhizobia were described in six genera within the α-subdivision of the proteobacteria (Fig. 1a). This phylogeny was based primarily on the 16S RNA gene supported by polyphasic analyses of metabolic activities and molecular markers in the cell wall. However, the reliance on 16S rRNA gene sequence analysis to determine the phylogenetic relationship between the rhizobia has been questioned, particularly by those who wished to preserve the *Agrobacterium* as an independent genus (Farrand et al. 2002). Currently the taxonomy of the Rhizobiaceae remains in a state of flux, the presence of large accessory genomes in the form of plasmids or transmissible genetic islands, containing the symbiotic genes that undergo transfers within and between species (Turner et al. 2002) means that lateral gene transfer is prevalent among the rhizobia. Moreover, recent analyses of 16S rRNA gene sequences of α--proteobacteria have demonstrated that the phylogeny derived using it is significantly different from that derived if the 23S rRNA gene or when the sequence of the region between the two RNA genes is used (van Berkum et al. 2003). In order to resolve these issues the sub committee on the taxonomy of *Agrobacterium* and *Rhizobium* of the International Committee on Systematics of Prokaryotes has suggested that the circumspection of new rhizobial species should not rely on DNA-DNA reassociation studies, a method that is considered to be the most significant test for species circumspection. Rather they proposed a new species definition based on

the analysis of several conserved functional genes such as gluA (glutamine synthase) and symbiosis genes such as nodA and nifH (Lindström and Martinez-Romero 2005; Saghal and Johri 2006). Furthermore, recent work by Moulin et al. (2001) has demonstrated that *Burkholderia* spp belonging to the β-proteobacteria were also capable of forming symbiotic nodules with leguminous plants. Subsequently other genera of both α and β-proteobacteria have been identified with the ability to form functional nodules. As a result the phylogenetic coherence of the rhizobia as α-proteobacteria has dissolved and currently the term rhizobia is used to collectively describe 44 species of plant nodulating bacteria dispersed among 11 genera of the α and β-proteobacteria (Fig. 1b), that because of the lateral transfer of the genes involved between different taxa, are in some cases phylogenetically only distantly related.

ENDOSYMBIOTIC N_2 FIXING BACTERIA

In recent years ^{15}N isotope dilution and ^{15}N natural abundance studies have provided strong evidence that diazotrophic bacteria such as *Gluconacetobacter diazotrophicus* and *Herbaspirillum rubrisubalbicans* that colonise some tropical grasses, especially sugarcane (*Saccharum* spp.) (Dong et al. 1994), wetland rice (*Oryza sativa*) and kallar grass (*Leptochloa fusca*) (Hurek et al. 2002) can provide some of N requirements of the plants from BNF. N_2 fixing bacteria that occupy intracellular spaces in the plant are described as endophytes, they are considered to play a major role in this process. Gene expression profiles of sugarcane colonised by *G. diazotrophicus* and *H. rubrisubalbicans* produced a number of candidate genes that may be exclusively or preferentially expressed during the N_2 fixing association. These data suggest that the host plant might be actively involved in the establishment of the interaction with *G. diazotrophicus* and *H. rubrisubalbicans* (Nogueira et al. 2001). A more recent study has looked for all expressed sequence tags (ESTs) preferentially or exclusively expressed in cDNA libraries constructed from sugarcane inoculated with *G. diazotrophicus* and *H. rubrisubalbicans*. This work identified EST candidates that may be involved in plant-bacteria signalling, suggesting that the initial steps of colonization are actively controlled by the plant in diazotrophic endophyte associations (Vargas et al. 2003). However, in the case of sugarcane at least, the amount of. N fixed is at least partly dependent on the plant genotype and its geographical location (James 2000). For example only a few Brazilian varieties have been shown to definitely fix N, whereas, studies from South Africa (Hoefsloot et al. 2005), Australia (Walsh et al. 2006) and Mexico (Munoz-Rojas and Caballero-Mellado 2003) have demonstrated no input from N fixation using ^{15}N natural abundance studies. As a result there has not been conclusive evidence that these plants are engaged in symbiotic partnerships with any bacteria (James 2000).

ASSOCIATIVE N_2 FIXING BACTERIA

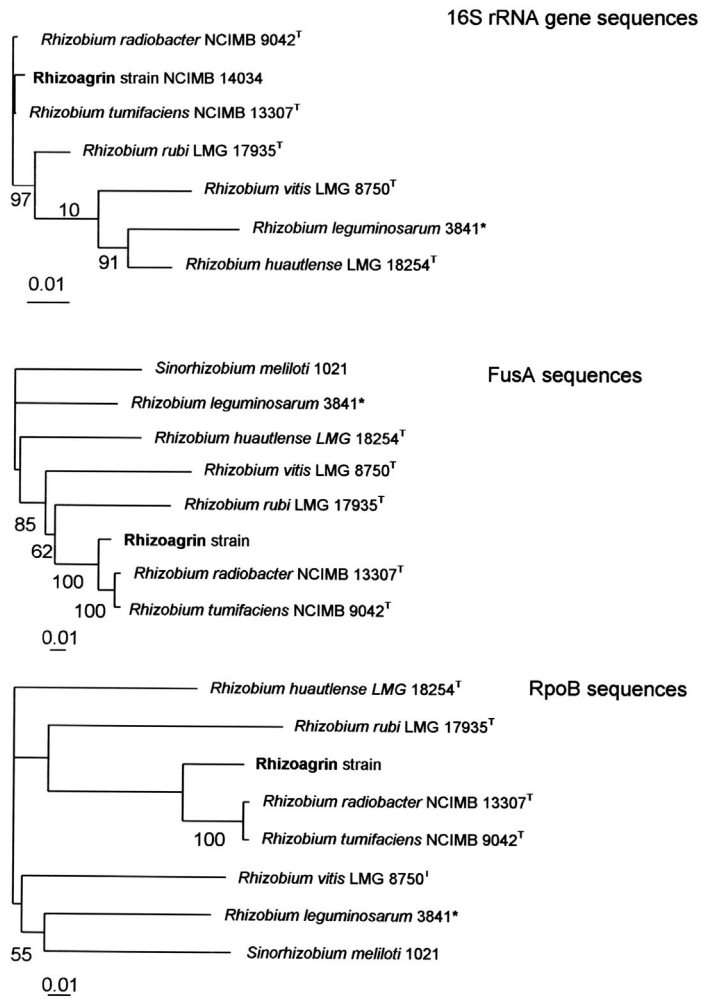
Many genera of diazotrophic bacteria such as *Azotobacter, Azospirillum, Herbaspirillum, Clostridium* and *Burkholderia* are commonly found as components of the soil flora. The contribution these bacteria make to the soil N budget is probably not significant under most conditions. Modelling to predict the amount of N fixed in the rhizosphere give values in the range of 0.2 to 4 kg N/ha (Jones et al. 2003). These amounts are small compared to inputs derived from symbiotic interactions and inorganic fertiliser. The most significant limitation according to this model appears to be the competition for C between N_2 fixing and non-N_2 fixing bacteria. Extensive research on the use of free-living bacteria as inoculants of non-leguminous crops to boost soil N has never produced a reliable effect despite a large number of field trials spanning several decades (Andrews et al. 2003). This is often because of confusion concerning the taxonomic status and activity of such inoculants, for example, 'Rhizoagrin' was an inoculant widely used in Russia and was claimed to give an increase in wheat yield equivalent to the application of 30 Kg N/ha. However, recent studies, using analyses of several conserved functional genes including the 16S rRNA, fusA and rpoB genes (Fig. 2), have demonstrated that the isolate used was genotypically very similar to the current *Rhizobium radiobacter* type strain (formerly *Agrobacterium radiobacter*) which does not fix N_2. It was apparent that the observed increases in cereal yields were via the production of plant growth promoting substances particularly gibberellic acid (Table 1) (Humphry et al. 2006). As a result of this and other studies it is now considered that the fixation of N_2 by these bacteria does not play a significant role in promoting plant growth, rather, when boosts in crop yield are observed, they are often a result of a number of factors including the production of plant hormones that lead to enhanced water and nutrient uptake and the suppression of soil pathogens (James 2000).

Frankia are Gram positive actinomycetes that often form symbiotic relationships with actinorhizal plants, they have also been identified as a small but significant component of the soil flora in agricultural soils used for permanent pasture and arable soil under rotation regimes with pasture (Garbeva et al. 2003). Studies on the effect of *Frankia* on non-actinorhizal plants suggested that soil containing *Frankia* had no significant effect on wheat (*Triticum aestivum* cv. pampa INTA), but did increase dry weight and total N in canola (*Brassica napus* cv. samurai) (Cusato & Tortosa 2000). However, this work has not been followed up in field trials, therefore, the effects of free-living *Frankia* cannot be attributed to enhanced soil N at this time.

CURRENT APPLICATIONS OF BNF IN AGRICULTURE

Despite the global decline in the use of legumes to enhance the N status of soils it remains as a key mechanism in a number of successful agricultural systems such as those of Australia and Brazil. In Australia, the exploitation of legumes using the

Fig. 2. Unrooted phylogenetic dendrograms based upon 16S rRNA sequences, fusA sequences and rpoB sequences. The comparisons were made using the Jukes and Cantor algorithm and the Neighbor-joining method, bootstrap confidence percentages were also calculated from 1000 replicate trees. The scale bar represents the number of nucleotide substitutions per 100 nucleotides. T denotes the type strain of the species. *Rhizobium leguminosarum* 3841 is currently undergoing genome sequencing, sequences marked * are available from www.sanger.ac.uk/Projects/R_leguminosarum.



ley-farming system, in which cereals are rotated with pasture legumes, became of significant agricultural importance during the 1940s. The amount of land under pasture, forage and pulse legumes in Australia has been estimated at 94 million hectares that fixes ~5 million tonnes of N per annum (Crews and Peoples 2004).

There is no doubt that the benefits in terms of N fixation have been substantial and enabled Australian agriculture to be globally competitive (Ridley et al. 2004). Both the legumes and their bacterial symbionts are exotic to Australia, therefore, for this agricultural system to be successful, effective rhizobial inoculants are required to ensure effective N fixation. Consequently, there has been extensive study to improve rhizobial inoculants for over 50 years (Brockwell 2004).

However, in recent years there have been concerns raised about the environmental impact that these farming systems have had, in particular, the degradation of the soil, the leaching of nitrate and the increase in crop losses due to the arrival of pests, disease and the emergence of herbicide tolerant weeds (Howieson et al. 2000). The major deleterious impacts that ley-farming has had on the fertility of the soil are firstly, salinisation that has arisen through the use of shallow rooting annual legumes that do not utilise the rainfall fully, and have resulted in the water table rising bringing

Table 1. Germination, seedling growth after 8 day incubation in the dark and shoot growth after 40 day incubation in the light of barley when treated with sterile water, growth medium or 'Rhizoagrin' strain culture or culture supernatant.

Treatment	Germination/seedling growth, room temperature in dark			Shoot growth, incubation 15-28°C in light
	Shoot length (mm)	Root length (mm)	Root branches (mm)	Shoot dry weight (g)
Sterile water	4.7	8.1	6.3	not determined
Yeast Mannitol Broth	4.1	7.7	6.4	0.48
'Rhizoagrin' culture supernatant	6.6	10.0	6.3	0.56
'Rhizoagrin' broth culture	6.7	10.6	6.2	0.61
LSD	0.93	1.65	0.6	0.044

salts to the surface (Howieson et al. 2000). The second is acidification, many soils in Australian agricultural systems are in any event ancient and significantly weathered resulting in them having low organic content and a significant degree of acidity. This can reduce the yield of many exotic pulses that were developed on the alkaline and fertile soils around the Mediterranean (Howieson et al. 2000). Acidification has been exacerbated by several factors, during N_2 fixation legumes take up an excess of cations and as a result excrete protons to counteract this (Raven et al. 1990). The result of this release can be significant and be strongly dependent on the legume species under cultivation, so for example, pH decreased by up to 0.85 pH units under lupin (*Lupinus angustifolius* L. cv. Gungurru) (Tang 1998) and the difference in acid production was two fold greater in soil under *Cicer arientinum* L. (Selection T1587) compared to *Pisum sativum* L. (cv. Wirrega) largely because the former releases organic acids rather than OH- during the uptake of NO_3- (Tang et al. 1999). Soil acidification can also arise through nitrification of the organic nitrogen as it is mineralised in the soil. Nitrate is highly mobile and will be rapidly leached from topsoil as it does so it increases the acidity of the soil. Soil acidity is also increased by the accumulation of organic matter in soil and an increase in the cation exchange capacity (Tang et al. 1997). As acidity increases, the solubility of toxic metal ions becomes more pronounced. This can result in decreases in crop yields by as much as 30% (Carr et al. 1991).

In South America, the problem of acid soils and other environmental stresses such as aluminium toxicity cause similar problems for farmers but reflect an attempt to utilise already marginal soils for agriculture rather than the Australian model where prolonged legume rotations have been instrumental in soil degradation. Over 25% of Brazil is comprised of a savannah called the 'Cerrados' subject to water stress, high temperatures and acidity with associated aluminium toxicity. Over 1 million hectares of this region are under common bean cultivation, typically by small scale farms that use minimal inputs (Mostasso et al. 2002). Yields are limited by N and P availability, often yields only average 587 Kg/ha and this has been attributed to poor N fixation.

CURRENT AND POTENTIAL ROLE OF RHIZOBIAL INOCULANTS IN AGRICULTURE

In both Australia and South America the continued success of BNF in farming systems requires flexibility in response to changing environmental, economic and biological pressures (Howieson et al. 2000). Problems with soil degradation through BNF may be ameliorated through broadening the diversity of legumes under cultivation. Whereas the continued cultivation of degraded or marginal soils also requires that appropriate strains of rhizobial inoculants are identified and deployed to maximise N fixation under such conditions (Graham and Vance 2000).

The introduction of a new legume into a soil is usually best accomplished if an effective rhizobial inoculant is also simultaneously added. One of the major problems with the use of BNF is that frequently inoculated seeds do not effectively nodulate, reducing the amount of N fixed by the crop. This can arise for a number of reasons including the inability of the inoculated bacterial strain to compete for nodule occupancy with resident soil rhizobia. Inoculants can also suffer high mortalities when introduced into a soil due to adverse environmental conditions.

Most soils have indigenous populations of rhizobia, even where both the legumes and their symbiotic bacteria are exotic. For example, the prolonged cultivation of a range of legumes has led to many Australian soils containing naturalised populations of rhizobia. These rhizobia are significant because they may nodulate any novel legume that is introduced to the soil. In some cases the resident population may be sufficient to effectively nodulate introduced legumes. Slattery et al. (2004) surveyed 50 soils around Southern Australia under a variety of legume crops for effective nodulation. A third of the soils had sufficient naturalised *Rhizobium leguminosarum* bv *viciae* to effectively nodulate faba bean (*Vicia faba* L), over 50% had sufficient to nodulate lentils (*Lens culinaris* L.), field pea (*Pisum sativum* L.) and two thirds gave effective nodulation of vetch (*Vicia sativa* L.). They demonstrated that the lowest populations of resident rhizobia were found in acid soils, whereas in alkaline soils the population size was often large enough to obviate the need for inoculation of the seeds.

Often, the naturalised or indigenous rhizobia are more competitive under soil conditions than any strain used as an inoculum, having adapted to edaphic conditions. Frequently, however, they also lose the ability to form efficient N_2 fixing symbioses with the legume. A recent study suggested that, in wild legumes at least, plants diverted fewer resources to nodules that were not occupied by effective N_2 fixing bacteria (Simms et al. 2006), while this would inhibit the spread of exploitative strains of rhizobia it would still result in less N fixation and reduced crop yields for the farmer. Studies on resident rhizobia have demonstrated that they can have a significant impact on the effectiveness of nodulation, for example, a commercial *R. leguminosarum* inoculant failed to outcompete naturalised rhizobia for nodule occupancy in the clovers *Trifolium alexandrinum, T. purpureum* and *T. resupinatum* (Denton et al. 2002).

The introduction of new inoculant strains requires some careful consideration, ideally they should be matched to both the soil conditions and the specific legume being introduced to the soil (Cummings 2005). Moreover, identifying appropriate strains that can effectively nodulate legumes in marginal soils offers the opportunity to extend the area of land suitable for agriculture. Releasing a new inoculant might be a problem if symbiosis between the new inoculant and the host plant is not optimal. Australian farmers are being advised to diversify the range of legumes that are incorporated into their rotations, as a result novel legumes from diverse geographic locations are being investigated for their potential. Work done on novel clovers (*Trifolium* spp.) demonstrated that there were significant obstacles to inoculation that correlated to their geographic origins and between annual and perennial clovers. Few inoculant strains were capable of forming effective symbioses across these barriers. As a consequence it was clear that development of effective inocula for new clovers that would not adversely affect N fixation in subterranean and annual clovers already widely used in Australian agriculture would be problematic (Howieson et al. 2005).

Recent evidence from both Australia and Brazil has clearly indicated that the saprophytic competence of the commercially used strains of rhizobial inocula varies between soils and, therefore, their effectiveness requires monitoring. This work was stimulated when field studies indicated that the one of the commercially used inoculant strains used in Brazil lost its ability to fix N_2 (Hungria et al. 2000). Commercial strains must be re-appraised to ensure that they will be effective when introduced into new soils, for example, the recommended inoculant for field pea strain SU303 used in south eastern Australia was investigated after it failed to maximise yields in Western Australia. It was found that other strains could be identified that gave more efficient N fixation in acidic soils and that this strain should be replaced by alternatives under these conditions (Evans 2005). Similarly the saprophytic competence of a current commercial inoculant, *Sinorhizobium meliloti* WSM826 used to nodulate lucerne (*Medicago sativa*) in mildly acidic soils was compared with a potential alternative strain WSM879. The latter nodulated 36% of lucerne seedlings compared to 27% when WSM826 was used. Both strains increased their nodulation efficiency two-fold when the soil was limed indicating that both strains have potential for development (Ballard et al. 2005).

In Brazil, as a result of the loss of N_2 fixing ability of the recommended inoculant of bean (SEMIA 4064) there has been a rational attempt to identify inoculant strains that can tolerate the harsh environmental conditions of Cerrrados soils and give an increase in crop yield. In many soils that have previously had beans cropped on them there are large indigenous populations of rhizobia that are capable of nodulating the common bean, but only inefficiently, requiring the addition of inorganic fertiliser to maintain yields (Vargas et al. 2000). However, in a study in which inoculant strains, very similar to *Rhizobium tropici*, were isolated from Cerrados soil and used as inoculants of bean crops, there was a statistically significant increase in yield despite the presence of high indigenous rhizobial populations (Mostasso et al. 2002).

An alternative strategy to identifying rhizobial strains adapted to soil conditions is to exploit the indigenous or naturalised rhizobia already present in the soil by developing varieties of crops that will nodulate promiscuously with these bacteria without the need for additional seed inoculation. This approach has been developed in Africa where promiscuously nodulating varieties of soya bean (*Glycine max* L. Merr.) have been developed. However, the efficacy of this approach is not clear cut, one study in Nigeria indicated that indigenous rhizobia were unable to meet the N requirements of the plant (Okogun and Sanginga 2003), whereas, another study in Zimbabwe demonstrated that some varieties of soya bean were capable of effectively fixing N without inoculation (Musiyiwa et al. 2005).

An additional problem with the use of inoculants is that unless the product is produced under strictly controlled conditions in 90% of cases it will be of little or no value in increasing crop productivity due to low rhizobial viability and contamination (Brockwell et al. 1995). This does little to instil farmers with confidence in their effectiveness. Inoculants are typically produced in a carrier material, such as peat, that is added directly to the seed or placed in the furrow prior to sowing, the latter method seems to be more effective in experiments conducted using inoculants of lentil (*Lens culinaris medik*) and chickpea (*Cicer arientum* L.) (Gan et al. 2005) . High quality inoculants employ a sterilised carrier and ensure a high number of viable rhizobia, whilst minimising contaminating organism. Successful inoculation requires large numbers of viable rhizobia per seed to ensure effective nodulation, as a result storage of the inocula can have a detrimental effect on its effectiveness. Longer shelf life are desirable for the manufacturers, however, under storage the viability of the inocula can decrease, particularly if stored at higher temperatures. Gemell et al. (2005) demonstrated that at refrigeration temperatures inoculant viability declined at 0.0005 log (10) units per day, however, the ability to form effective nodules

did not diminish even after 18 months storage. Inoculant manufacture is under strict legislative controls in some countries to ensure the quality of the inoculant (Bullard et al. 2005). However, as was recently reported many inoculants remain of a poor quality (Catroux et al. 2001).

FUTURE POTENTIAL FOR BNF IN AGRICULTURE

The future for BNF in agriculture appears to be uncertain, the burgeoning population and decreasing area of cultivatable land has led some to suggest that it will not be a technology that will provide sufficient protein to meet the growing demand over the next century (Jenkinson 2001). In contrast, others argue that it may play a significant role if there is the political and economic will to affect changes in the way food is produced and land is used globally (Crews and Peoples 2004). The role of scientific innovation will be to identify how the exploitation of BNF may be optimised. The development of technologies that enable the more efficient and cost effective use of BNF may increase its attractiveness to farmers, for example, increasing BNF in rice production has been under scrutiny for a number of years. The surface soils and the rhizosphere under rice cultivation are populated with a number of cyanobacterial, heterotrophic and photosynthetic diazotrophic bacteria. The quantification of the contribution of these bacteria to the N budget of the plant is problematical, however, estimates of 30 kg N/ha (Ladha and Reddy 2001) suggest a modest but significant contribution that may offer scope for some improvement with appropriate farming practices. The use of green manures such as the water fern *Azolla* and legumes have also been widely used and can fix significantly more N than that of the associative diazotrophic bacteria. Unfortunately, *Azolla* despite reducing the requirement for inorganic N addition by 50% is subject to temperature sensitivity and maintenance of *Azolla* inocula between crops is difficult. Therefore, developing new strains that are more robust to temperature and devising technologies that allow large scale spore production to improve inoculation of rice fields may enhance the use of this approach. The legume *Sesbania rostrata* can replace the requirement for inorganic N addition in rice cultivation but competes with the rice and without appropriate uptake of agricultural practices that enable this problem to be circumvented will not be widely adopted by farmers (Choudhury and Kennedy 2004).

An alternative approach would be to exploit advances in molecular biology that may enable more widespread adoption of BNF in agriculture. For example, by enabling the development of elite highly effective strain of genetically modified rhizobia to improve N fixation in legume rotations or by developing novel varieties of non-leguminous crop that may be capable of fixing N using either rhizobia or endophytic diazotrophic bacteria. The use of genetically modified bacteria as inoculants to improve the fixation of nitrogen has been recently reviewed (Hirsch 2004). In drawing together the data from a number of trials using genetically modified rhizobia it is clear that as with conventional inocula the persistence of such bacteria in the soil was limited, particularly when there were large indigenous rhizobial populations. Moreover, saprophytic competence is probably a multifactorial trait and attempts to improve the persistence of such inocula as well as select for high levels of N_2 fixing efficiency are probably economically unrealistic with present technology.

Perhaps the most important goal of research on the N_2 fixing bacteria is to determine whether it would be possible to provide non-leguminous plants with this ability. In particular, to enable the agriculturally most significant cereal crops, rice, wheat and maize to fix N. There are essentially two approaches to tackling this problem. The first is to exploit N_2 fixing bacteria as inoculants that will colonise these crops and exhibit N fixation *in planta*. As we have already seen there is evidence, most convincingly from the studies on sugar cane, that some plants can obtain substantial amounts of fixed N from endosymbiotic bacteria (James et al. 2001). The second approach is to attempt to genetically engineer such crops so that they contain active N_2 fixing systems, however, this approach presents some extremely difficult technical challenges (Dixon et al. 1997).

Recently there have been a number of studies that suggest an opportunity for utilising endosymbiotic bacteria in cereal production. The first report of nitrogen fixation in wheat (*Triticum aestivum* L.) using a bacterial inoculant *Klebsiella pneumoniae* has recently been published. The activity was limited to a single wheat cultivar (Trenton), however, N deficiency was relieved and total plant N increased significantly (Iniguez et al. 2004). Further analysis of this cultivar may identify those factors that enable this interaction to occur and perhaps allow them to be incorporated into other wheat cultivars.

An interesting recent development has been the report that *Gluconacetobacter diazotrophicus*, a N_2 fixing bacteria responsible for endophytic colonisation of sugar cane, can using a novel inoculation technique colonise the roots of maize (*Zea mays* L.), rice (*Oryza sativa*), wheat (*Triticum aestivum*), rape (*Brassica napus*), tomato (*Lycopersicon esculentum*) and clover (*Trifolium repens*) forming membrane bounded vesicles that express N_2 fixing activity (Cocking et al. 2006). Whether these N fixing vesicles have the potential to improve the N content of the plants or eliminate N deficiency has yet to be determined.

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